Chapter V

Estrogenic Effect of *Pueraria mirifica* Phytoestrogens on the Decrease of Gonadotropin Levels in Aged Menopausal Monkeys

Abstract

To investigate the long-term effect of *Pueraria mirifica* (PM) on the secretion of gonadotropins and estradiol in aged animals, nine menopausal cynomolgus monkeys were used. They were divided into 3 groups and were fed with 10, 100, and 1,000 mg/day of PM, respectively. Blood samples were collected every 5 days during the 30 days of pre-treatment, 90 days of PM treatment, and 60 days of post-treatment, respectively and assayed for serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and estradiol. The results showed that PM-10 induced the decrease of FSH levels on days 15 – 90 during the treatment period in 1/3 of monkeys. PM-100 and PM-1,000 decreased FSH levels throughout the treatment period. After the cessation of PM treatment, the decrease of FSH levels was maintained for 5 and 10 - 20 days in PM-100 and PM-1,000, respectively, and the levels were rebounded in all groups thereafter. PM-10 decreased LH level throughout the treatment period in 1/3 of monkeys and returned to the pre-treatment levels immediately after the cessation of PM treatment. PM-100 and PM-1,000 prominently decreased LH levels between days 10 – 90 during treatment period and, the effect was maintained until days 15 – 25 and days 20 – 30 during the post-treatment period by PM-100 and PM-1,000, respectively. LH levels showed the rebounded levels after returning to the pre-treatment levels in a dose dependent manner. Serum estradiol levels tended to decrease during the treatment period in all

groups. It is concluded that daily intake of PM suppresses gonadotropin levels in aged menopausal monkeys dose dependently. The decrease of hormonal levels can be recovered with the durations dependent on doses. The higher dose takes the longer time to recover. From this study, it is suggested that PM can be used as an alternative medicine in aged menopausal women for the estrogen effect, because the effect is reversible after stop using.

Key words: *Pueraria mirifica*, phytoestrogen, gonadotropin, estradiol, menopause monkey

5.1 Introduction

The white kwao krua, *Pueraria mirifica* (PM), is a Thai medicinal herb that belongs to the family Leguminoceae. Its tuberous root was proved to be extremely rich in phytoestrogens of isoflavone group (Muangman and Cherdshewasart, 2001). In addition, it consists of small amount of other groups of phytoestrogens including coumestrol (Ingham et al., 1986a, 1989), miroestrol (Pope et al., 1958), puerarin (Ingham et al., 1986b), deoxymiroestrol, and kwakhurin (Chansakaow et al., 2000a, 2000b). From the *in vitro* study, miroestrol showed a high estrogenic potency when assayed by the immature mouse uterine weight and rat vaginal cornification tests (Pope et al., 1958; Jones and Pope, 1960). Miroestrol treatment increased uterine weight in immature female mice (Jones and Pope, 1960) and produced the cornification of the vaginal epithelium in ovariectomized-adrenalectomized rats (Pope et al., 1958). Nowadays, PM become of widely interest in biological research by its estrogenic properties. Our prior studies showed that a single dose of 10, 100, and 1,000 mg/day of PM feeding prolonged the menstrual cycle length in adult cyclic

cynomolgus monkeys (Trisomboon et al., 2004). Moreover, the long-term treatment for 90 days suppressed serum levels of gonadotropins and ovarian hormones in dose dependence. The monkeys fed with the highest dose (1,000 mg/day) showed amenorrhea symptoms (Trisomboon et al., 2002). From the clinical trial in women, long-term consumption of PM reduced the postmenopausal symptoms including hot flush, sleep disorder, and skin dryness (Muangman and Cherdshewasart, 2001). The previous studies, however, have reported no data of the effect of PM on reproductive hormones in aged menopausal women with an ovarian estradiol deficiency. Actually, a number of aged menopausal women who turned to use natural estrogenic substances, instead of synthetic estrogen, for their menopausal symptoms, are particularly increased. It means that phytoestrogens are considered to be effective.

Several studies have shown that daily intake of phytoestrogen isoflavones from soy affected the reproductive function and altered the secretion profile of reproductive hormones in both premenopausal (Baird et al., 1995; Duncan et al., 1999) and postmenopausal women (Murkies et al., 1995). Additionally, the epidemiological studies showed the lower levels of estrone and estradiol in postmenopausal Japanese women who consumed high amounts of soy isoflavones (Shimizu et al., 1990).

Therefore, it is of interest to investigate the long-term effect of PM treatment on serum levels of gonadotropins and estradiol in aged women. The study on the long-term effect of PM on humans is, however, very difficult to follow-up because of the uncontrolled diet. Female cynomolgus monkeys (*Macaca fascicularis*) were, therefore, used as a model in this study, because of their similarity in the hormonal patterns and reproductive system to those of humans (Krajewski et al., 2003).

5.2 Materials and Methods

5.2.1 Animals

Aged menopausal monkeys (*Macaca fascicularis*, n=9) with a complete cessation of menstruation for at lest 1 year and weighing 4.0 - 6.5 kg were selected. Menopausal state of the monkeys was confirmed and checked daily by vaginal swabbing method before and during treatment. The monkeys were housed separately in an individual cage at the Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. Lighting conditions of the animal room were controlled (12 : 12 h light to dark cycle). Temperature and humidity fluctuated slightly depending on the season. The monkeys were fed daily monkey chow (Pokaphan Animal Feed Co., Ltd., Bangkok, Thailand) in the morning (09:00 – 10:00 h) and supplemented with fresh fruits in the afternoon (14:00 – 15:00 h). The experimental protocol was approved by the ethics committee in accordance with the guide for the care and use of laboratory animals prepared by the Primate Research Unit, Chulalongkorn University.

5.2.2 Experimental Design

Nine aged menopausal monkeys were divided into three groups. Monkeys in each group (n = 3) were fed with the suspension of PM at doses of 10, 100, and 1,000 mg/individual/day (abbreviated as PM-10, PM-100, and PM-1,000, respectively, hereafter) between 08:00 – 08:30 h. The treatment schedule was separated into 3 periods: pre-treatment, treatment, and post-treatment. During the pre-treatment and post-treatment periods, monkeys were fed daily with 5 ml of distilled water for 30 and 60 days, respectively. During the treatment period, monkeys were fed daily with the suspension of PM for 90 days. Three-ml blood samples were collected from the femoral vein without anesthetization between 08:30

– 09:30 h every 5-day and then were centrifuge at 1,700 x g, 4 ° C for 20 minutes and sera were stored at –20 ° C until hormonal assays were performed.

5.2.3 Preparation of the Suspension of *Pueraria mirifica*

The fresh tuberous roots of PM were sliced, dried in hot air oven at 70 ° C, and subsequently ground into powder at size of 100 Mesh. Then, the stock of its powder was kept in the dark desiccator before the preparation of its suspension. PM powder was suspended into distilled water and then kept in a dark bottle at 4 ° C until the feeding time.

5.2.3 Hormonal Analyses

The serum samples were analyzed for FSH and LH levels using a heterologous RIA system described previously (Hodgen et al., 1976). Iodinated preparations were rat NIDDK-rat FSH -I-5 and rat LH-I-5. The anitisera were anti-ovine FSH (NIDDK-H-31) and anti-ovine LH (YM#18). Antiserum against ovine LH (YM#18) was kindly provided by Dr.Y.Mori (University of Tokyo, Tokyo, Japan). After extraction of ether, serum level of estradiol was determined by double-antibody RIA with ^{3}H-labeled radioligands as described in the established method of World Health Organization (WHO) (Sufi et al., 1986).

5.2.4 Statistical Analyses

Serum hormonal levels of three monkeys in each group were expressed as mean $\pm$ S.E.M. Analysis of variance (ANOVA) followed by the LSD test was applied to determine the significant difference among those three periods and among all three groups. Differences were considered to be significant at $P < 0.05$.

5.3 Results

5.3.1 Basal Levels of Serum Gonadotropins and Estradiol in Aged Menopausal Monkeys

Menopausal state of monkeys in this study was confirmed by the low levels of serum estradiol (14.71 ± 1.18 pg/ml) and the high levels of both serum FSH (3.81 ± 0.61 ng/ml) and LH (5.85 ± 0.80 ng/ml) throughout the 30 days of pre-treatment period. When we compared these levels in aged menopausal monkeys with those normally cycling monkeys in the early follicular phase of the menstrual cycle in our colony, the estradiol levels were lower, but the FSH and LH levels were higher than the latter (27.54 ± 4.31 pg/ml for estradiol, 1.60 ± 0.33 ng/ml for FSH, and 0.51 ± 0.03 ng/ml for LH).

5.3.2 Changes in Serum Levels of Gonadotropins and Estradiol in Monkeys Treated with PM

Changes in serum levels and concentration patterns of gonadotropins during the treatment and post-treatment periods in monkeys treated with PM-10, PM-100, and PM-1,000 compared with the pre-treatment levels are shown in Figures 5.1 - 5.3, respectively.

In monkeys treated with PM-10, as shown in Figure 5.1, FSH levels decreased on days 10 - 15 in monkey nos. 58 and 85 and on days 15 - 90 in monkey no. 11 during the treatment period. Serum levels of FSH in all monkeys, however, tended to recover the pre-treatment levels during the treatment period and rebounded during the post-treatment period. In all monkeys treated with PM-100, FSH levels decreased prominently on days 5 – 90 during the treatment period. Serum levels of FSH remained low until the first 5 days of the post-treatment period and rebounded thereafter. In monkeys treated with PM-1,000, FSH levels decreased

prominently on days 5 - 90 during the treatment period and kept the low levels until days 10 – 20 of the post-treatment period before returning to the pre-treatment levels. Interestingly, during the post-treatment period, FSH levels in monkeys treated with PM-1,000 showed a rebound slower than the monkeys treated with PM-100 and PM-10, respectively.

As shown in Figure 5.2, LH levels in monkey no. 11 treated with PM-10 were decreased prominently on day 5 during the treatment period, and were kept low thereafter. In monkey nos. 58 and 85 treated with PM-10, LH levels did not significantly change from the pre-treatment levels. In monkeys treated with PM-100 and PM-1,000, LH levels decreased prominently within 10 days from the beginning of the treatment period. During the post-treatment period, the levels remained low until the first 15 - 25 days for the PM-100 and the first 20 - 30 days for PM-1,000. Similar to the response of FSH, LH levels during the post-treatment period increased to the pre-treatment levels and rebounded afterward in all treatment groups.

As shown in Figure 5.3, estradiol levels in monkeys of all groups tended to be decreased during the treatment period and increased to the pre-treatment levels in monkeys treated with PM-10 and PM-1,000. However, all monkeys treated with PM-10 and PM-100 did not recover to the pre-treatment levels during the post-treatment period except monkey no. 42.

5.3.3 Comparing Effects of the Different Doses of PM on Gonadotropin and Estradiol Levels

There were high variations of FSH and LH levels during the pre-treatment period of monkeys in each group because of differences in the timing to enter the menopausal state. The monkeys entered the menopausal state earlier (> 5 yrs) had the higher level of FSH (P = 0.01) and LH (P = 0.01). Those in later entering monkeys (< 5 yrs). Estradiol levels, however, did not significantly differ between the

two groups of monkeys (P = 0.95), as shown in Figure 5.4. Thus, to compare the potential effect of various doses of PM on hormonal levels, serum levels of hormones were adjusted to percent changes from the pre-treatment levels, arbitrarily assigned at 100 and pooled the data of that three monkeys together.

As shown in Figure 5.5, during the treatment period, FSH levels decreased prominently to 37.04, 19.33, and 6.69 % of the level in the pre-treatment level on day 10 for PM-10, PM-100, and PM-1,000, respectively. FSH levels remained low in PM-100 and PM-1,000 (37.04 – 244.67 % for PM-10, 7.36 – 19.33 % for PM-100, and 3.09 – 20.15 % for PM-1,000) throughout the treatment period. During the post-treatment period, FSH levels increased and rebounded on days 10 and 20 for PM-100 and PM-1,000, respectively. LH levels showed a prominent decrease, between days 10 – 90 during the treatment period, to 51.07 – 128.68 % for PM-10, 22.70 – 77.32 % for PM-100, and 18.86 – 32.98 % for PM-1,000. LH levels rebounded on days 5 and 45 of the post-treatment period for PM-10 and PM-100, respectively. The LH levels in PM-1,000 group, however, were kept lower than the pre-treatment levels until day 60 of the post-treatment period.

Estradiol levels decreased within days 10 – 90 during the treatment period and increased to the pre-treatment levels during the post-treatment period in all monkey groups. The suppression of PM phytoestrogens on estradiol levels did not appear dose dependent .

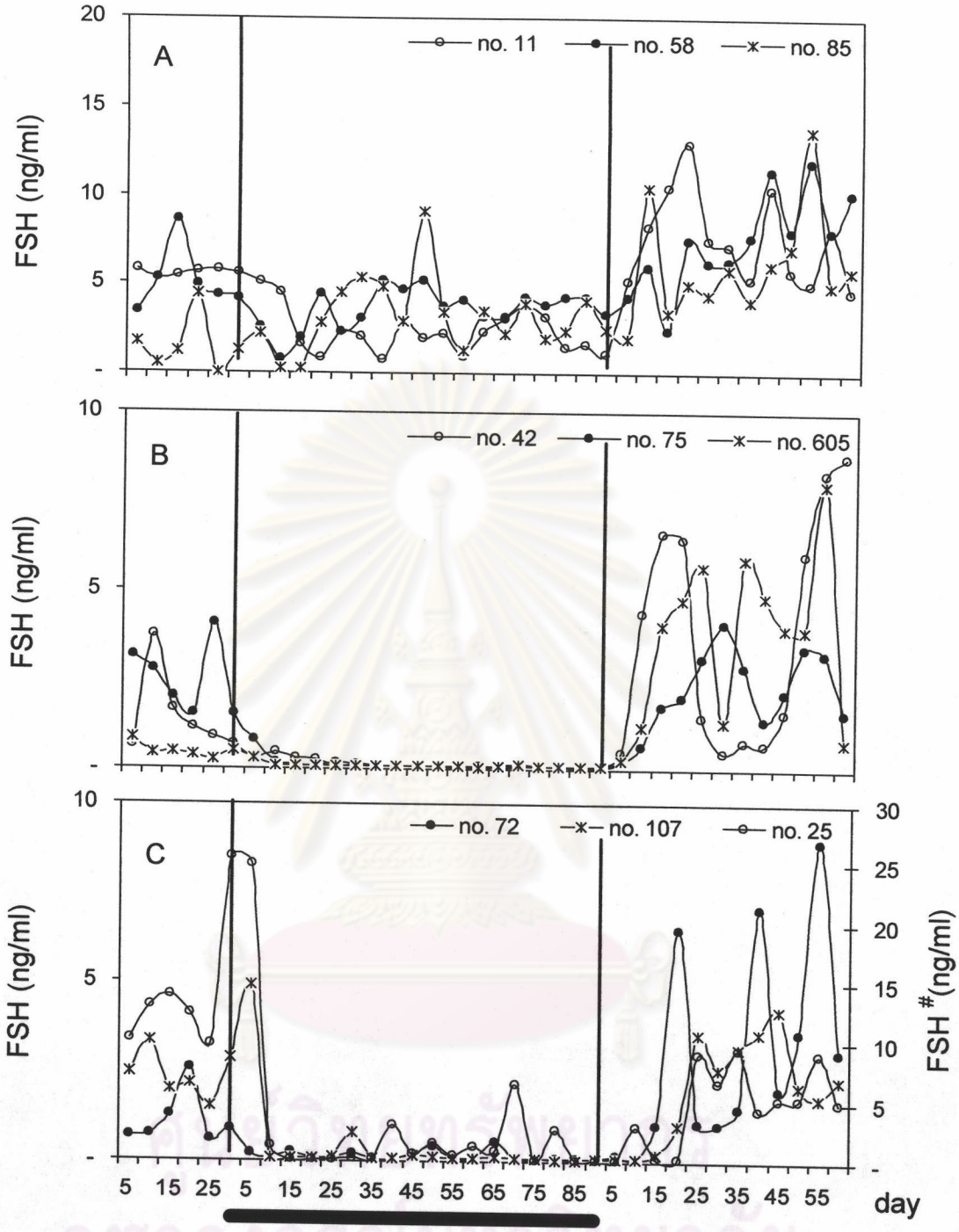

Figure 5.1 Changes in FSH levels during the pre-treatment, treatment, and post-treatment periods in aged menopausal monkeys treated with 10 (A), 100 (B), and 1,000 (C) mg/day of PM. The vertical lines indicate the border between periods. A black horizontal line indicates treatment period. # indicates serum FSH levels of monkey no. 25

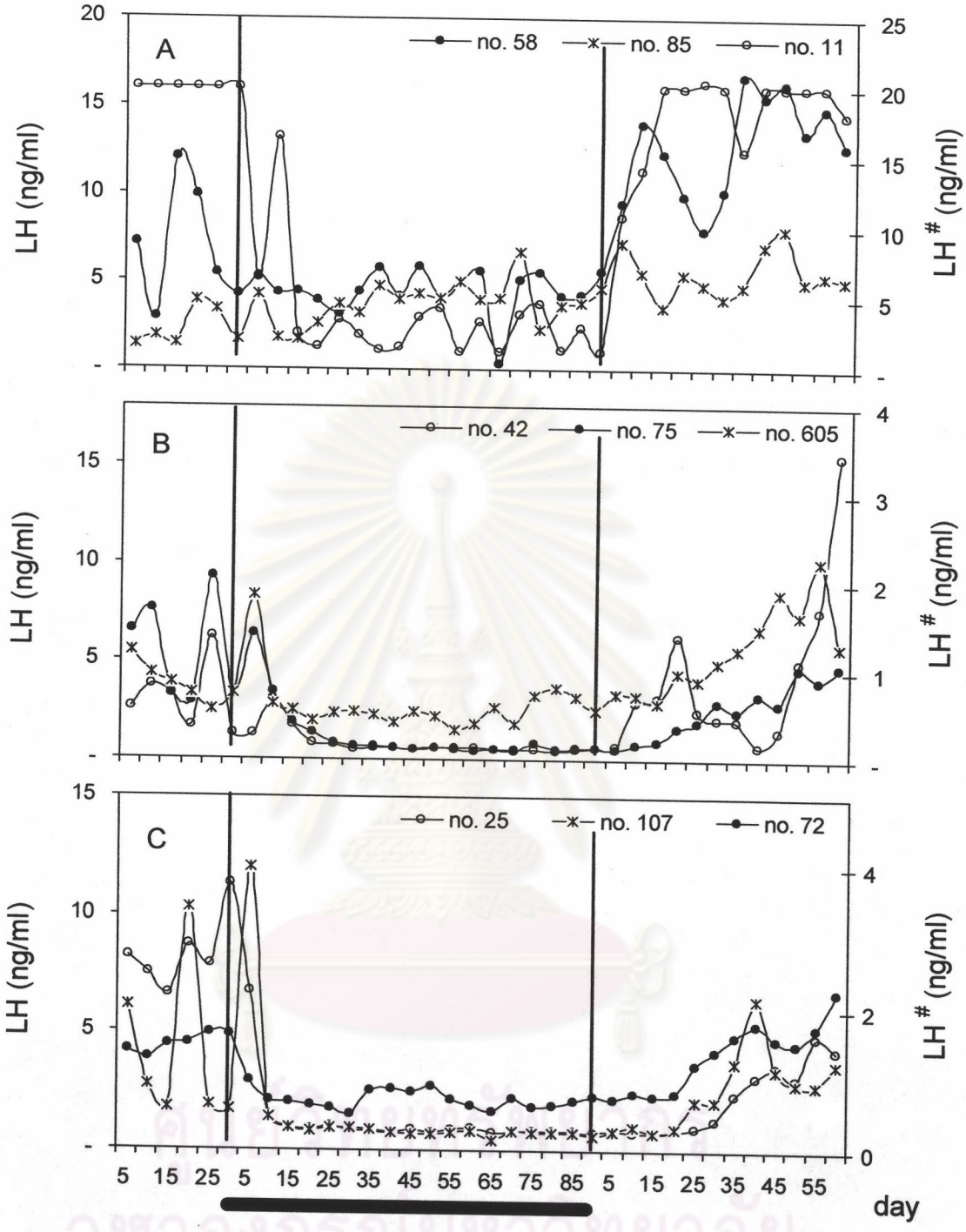

Figure 5.2 Changes in LH levels during the pre-treatment, treatment, and post-treatment periods in aged menopausal monkeys treated with 10 (A), 100 (B), and 1,000 (C) mg/day of PM. The meanings of the vertical and black horizontal lines are the same as those given in Figure 5.1. # indicates serum LH levels of monkey nos. 11, 605, and 72.

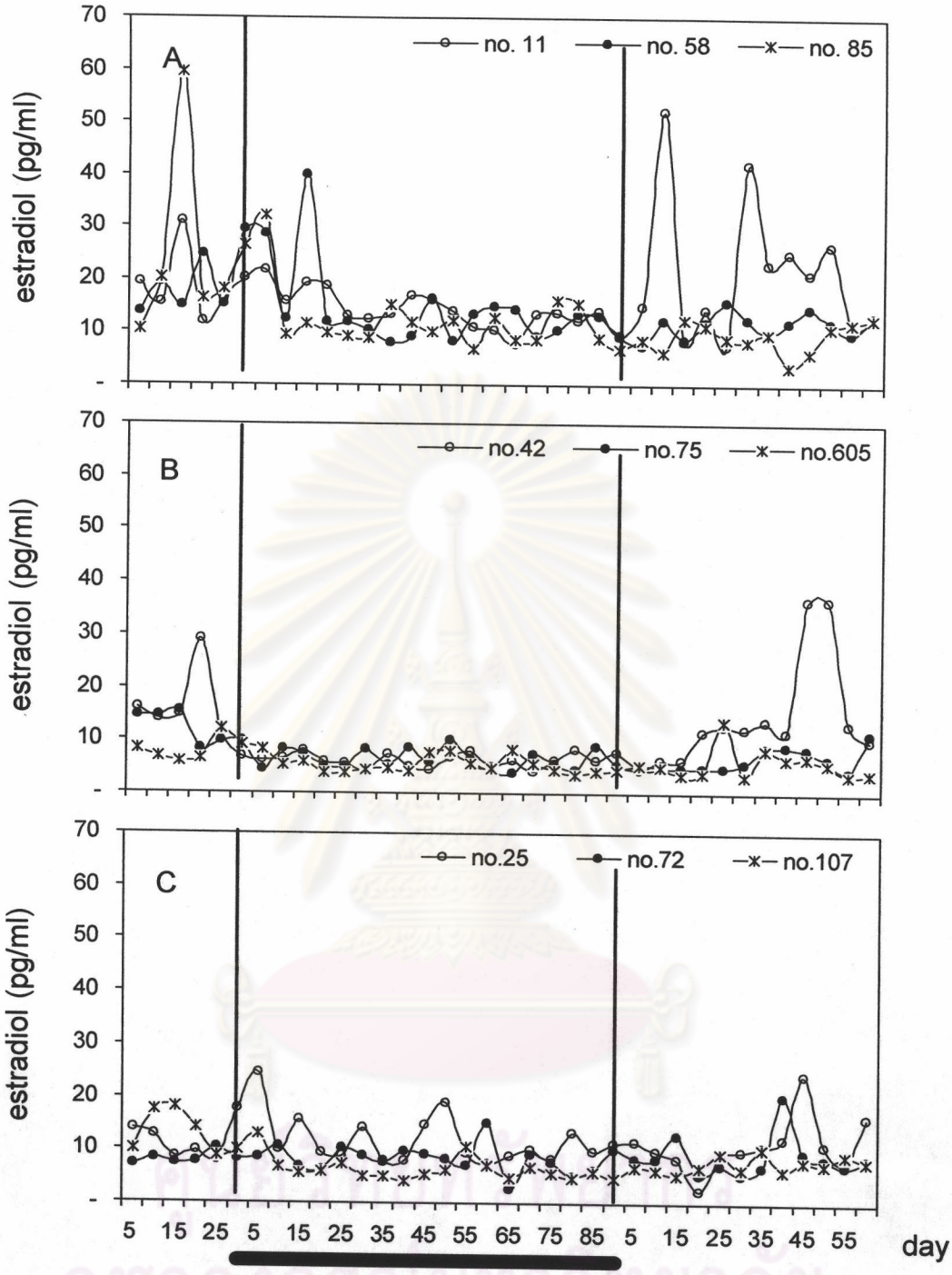

Figure 5.3 Changes in estradiol levels during the pre-treatment, treatment, and post-treatment periods in aged menopausal monkeys treated with 10 (A), 100 (B), and 1,000 (C) mg/day of PM. The meanings of the vertical and black horizontal lines are the same as those given in Figure 5.1.

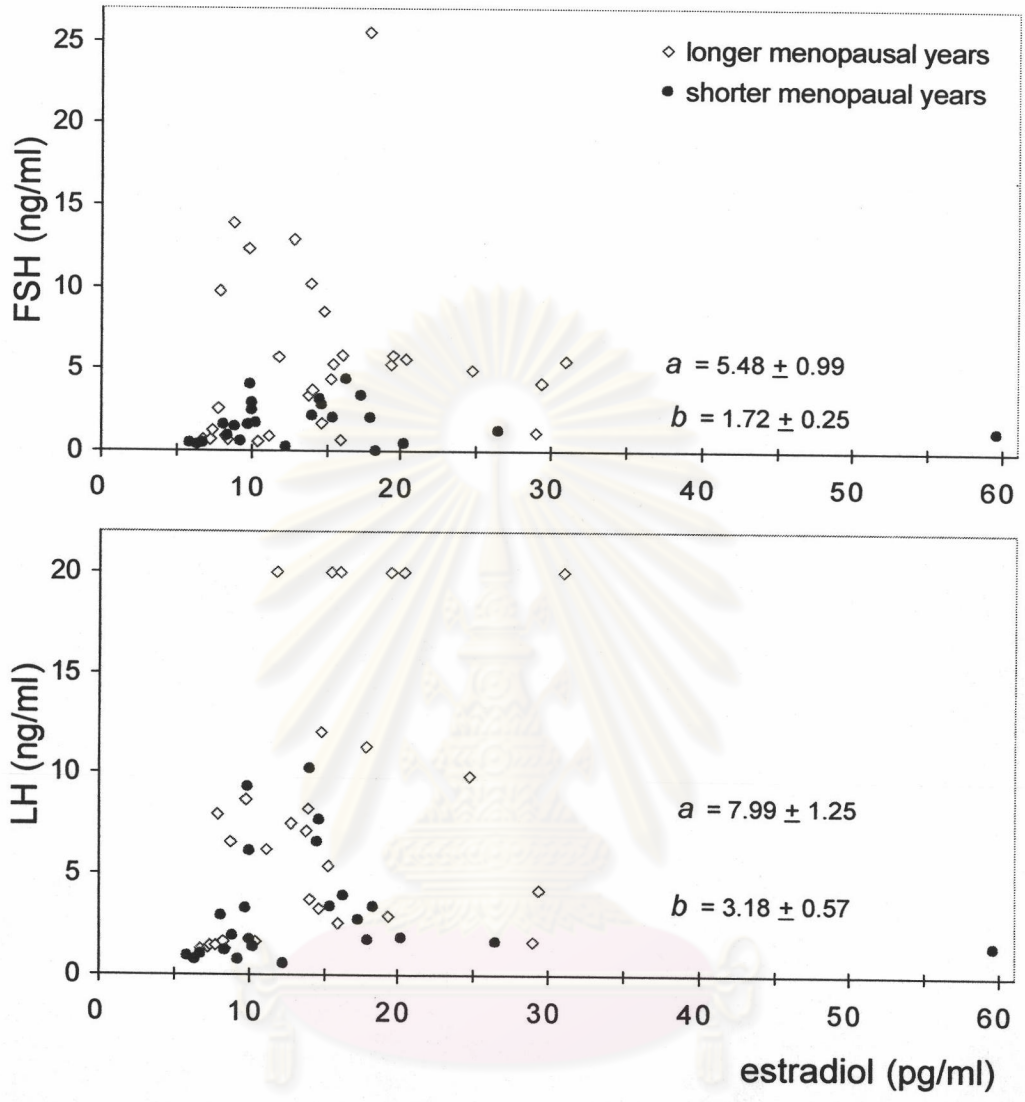

Figure 5.4 Basal level of gonadotrophins (FSH and LH) and estradiol in aged monkeys, whose menopausal lives are longer, and shorter than 5 years. Their means and S.E.M. were indicated by *a* and *b*, respectively.

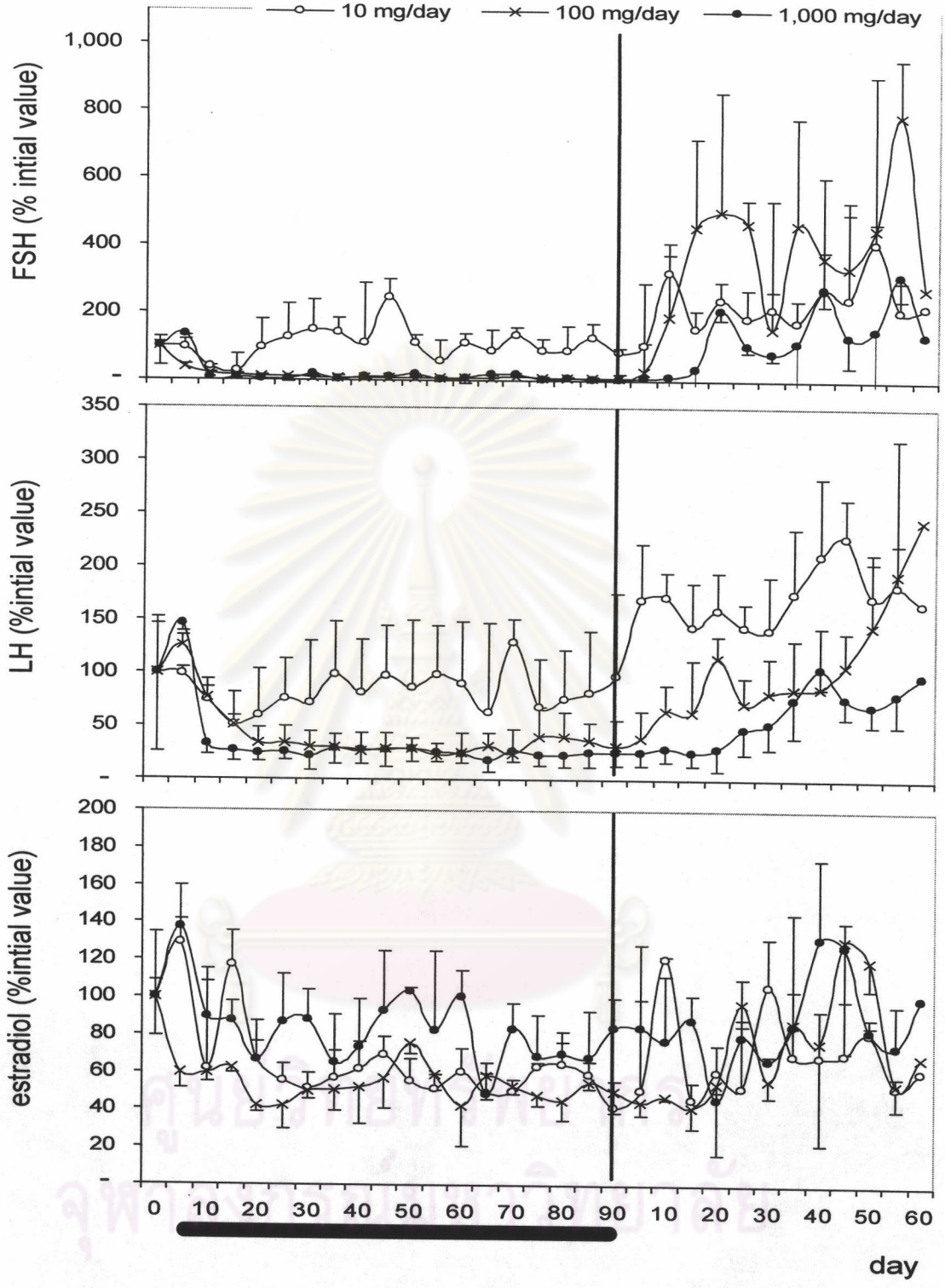

Figure 5.5 Serum levels of gonadotropins and estradiol during the treatment and post-treatment periods were standardized by the percent changes against the pre-treatment levels. A black horizontal line indicates the treatment period. Each of the plots expressed the mean ± S.E.M.

5.4 Discussion

It has long been recognized that estrogen deficiency due to the cessation of ovarian function in aged menopausal monkeys is associated with a significant rise in pituitary gonadotropin levels, similar to those in postmenopausal women (Park et al., 2002; Woller et al., 2002). Loss of ovarian estrogen with menopause results in the malfunction of the negative feedback mechanism on the hypothalamus and pituitary and, thus, both of FSH and LH levels are increased. In this study, we found that oral administration of PM induced a prominent decrease in both of FSH and LH levels in aged menopausal monkeys in a dose-dependent manner. Comparing the response of FSH and LH levels to different doses of PM, the higher dose exhibited the higher potency in the decrease of FSH and LH levels.

PM is known to contain many kinds of phytoestrogens (Pope et al., 1958; Ingham et al., 1986a, 1986b, 1989; Chansakaow et al., 2000a, 2000b; Muangman and Chershewasart, 2001), mainly isoflavones, which present nonsteroidal structures similar to those of estrogens. They can bind to estrogen receptors and exert estrogenic effect in mice (Jones and Pope, 1960), rats (McGarvey et al., 2001), and humans (Cassidy et al., 1994, 1995; Duncan et al., 1999). Previous studies investigating the potential action of phytoestrogen, genistein, on the hypothalamus and pituitary axis in ovariectomized rats and demonstrated that genistein administration blocked the GnRH-induced rise of LH in ovariectomized rats (Faber and Hughes, 1991; Hughes et al., 1991). Coumestrol administration led to the decrease of the GnRH pulse generator frequency, reduced pulsatile secretion of LH in addition to suppression on pituitary LH response to GnRH priming in ovariectomized rats both *in vivo* and *in vitro* studies (McGarvery et al., 2001).

Concurrently, studies on exogenous estrogen administration showed the direct effect of the negative feedback mechanism. Estradiol benzoate reduced LH release

caused by reducing the sensitivity of gonadotrope to GnRH stimulation without altering the GnRH secretion pattern in ovariectomized rhesus monkeys, suggesting that the target site of estrogen is the pituitary level (Nakai et al., 1978; Pau et al., 1990). In addition, administration of estradiol benzoate using push-pull perfusion decreased pulse amplitude and basal release of GnRH and reduced LH levels in ovariectomized rhesus monkeys, suggesting that the target site of estrogen in the hypothalamus level (Changthammakun and Terasawa, 1993; O'Byrne et al., 1993). In the present study, although we could not conclude that PM treatment affects at the hypothalamus level, we could assume that PM has an estrogenic effect to decrease in gonadotropins by the negative feedback mechanism at the hypothalamus and pituitary. The conclusion that the estrogenic effect of PM phytoestrogens to suppress gonadotropin levels was confirmed by studies on the effect of synthetic estrogens, such as conjugated estrogen, estradiol valerate, and others (Varma et al., 1985; Casson et al., 1997; Bray et al., 2001).

The deficiency of ovarian function in postmenopause results in the decrease of the secretion of ovarian estradiol. Aged menopausal monkeys in this study have the lower levels of serum estradiol than those in normal cyclic monkeys in the early follicular phase. In the responding to the long-term treatment of PM, serum levels of estradiol decreased during the treatment period and returned to the pre-treatment levels after the cessation of PM treatment in all monkey groups. Estradiol in menopausal individuals mainly comes from the peripheral conversion of androstenedione via estrone and androstenedione via testosterone. There are *in vitro* studies demonstrating that the genistein and coumestrol reduced these conversions (Makella et al., 1995; Whitehead et al., 2002). These findings seem to imply that PM phytoestrogens may have a direct effect on estradiol production by the conversion of other steroid hormones, not depended on the decreased

gonadotropins through the hypothalamus-pituitary-gonadal axis. Nevertheless, the additional studies for the further understanding have to be done.

Estrogenic effect of soy phytoestrogens on serum levels of reproductive hormones has also been studied in postmenopausal women. The daily intake of high amounts of soy containing 165 mg isoflavones for 4 weeks induced the slight decrease in circulating levels of FSH, LH, and estradiol in postmenopausal women (Baird et al., 1995). At lower doses of isoflavones, 7.1 - 132 mg/day for 3 months, however, could not change serum FSH or LH levels (Duncan et al., 1999). The same result was obtained in another study that is, the daily intake of 56 and 90 mg of isoflavones for 3 or 6 months, could not change serum levels of FSH, LH, or estradiol in postmenopausal women (Persky et al., 2002). Muangman and Cherdshewasart (2001) analyzed the content of isoflavones in PM, and proved that PM in the same lot used in the present study contains 1.691 mg total isoflavones/g dried powder. Thus, the isoflavones content in the present PM treatment of 10, 100, and 1,000 mg/day are less than those contained in soy used in other studies (Baird et al., 1995; Duncan et al., 1999; Persky et al., 2002). However, the lowest dose of PM (10 mg/day), containing 0.0169 mg isoflavones, could clearly suppress serum FSH and LH levels in aged monkeys. It can, therefore, postulate that the potency of PM phytoestrogens is stronger than soy phytoestrogens under the same quantity in isoflavones. From *in vitro* study, coumestrol and miroestrol had relative molar binding affinities to estrogen receptors as high as 5% of estradiol. Meanwhile, relative molar binding affinities of daidzein and genistein to estrogen receptors are between 1- 0.05 % (Shutt and Cox, 1972). It is possible that the stronger potency of PM than soy is due to the presence of other kind of phytoestrogens in PM, e.g. coumestrol and miroestrol.

The time course of PM function is worth while considering. Suppressive effect of PM on serum levels of gonadotropins was observed on days 10 - 90 during the treatment period. Pharmacokinetics studies showed that after ingestion of soy

isoflavones, the elimination half-life of genistein and daidzein are 7 and 4 hours in premenopausal women and 4 and 3 hours in men (Lu and Anderson, 1998). Other studies also indicated that half-lives of genistein and daidzein are 5.5 and 7.4 hours in premenopausal women (Setchell et al., 2003) and 8.3 and 5.8 hours in men, respectively (Watanabe et al., 1998). From these evidences, it can assume that at the initial feeding, concentrations of phytoestrogens in blood circulation did not reach to the threshold level of response. Although no physiological response could be observed, the biotransformation of phytoestrogens should have occurred and the metabolites were excreted through the urine. After a daily treatment of PM for approximately 10 days, the concentrations of phytoestrogens in the blood circulation were accumulated to reach the threshold concentrations. The full-physiological response occured in all monkeys, e.g. the decrease of FSH and LH levels. This study also found the latency period of the recovery of gonadotropin levels during the post-treatment period, in a dose-dependent manner. After the cessation of PM treatment, FSH and LH levels remained low for several days before recovery to the pre-treatment levels. FSH and LH levels were rebounded abruptly after the cessation of PM-10, on the other hand it took a delaying time for the rebound action in the higher doses (PM-100 and PM-1,000). The evidence of rebound effect was also found in the other study (Gianotti et al., 2003). A single subcutaneous injection of GnRH antagonist, that is the competitive inhibitor of GnRH receptors, reduced serum FSH and LH levels within 6 – 48 hours and rebounded serum LH levels at 96 hours. From this study, we can postulate that there is the enhancement of hypothalamic GnRH drive after the relief of its antagonist (Gianotti et al., 2003). Accordingly, we assume that the rebound of gonadotropin levels during the post-treatment period is caused by the increased responsiveness of gonadotrope to GnRH.

In summary, the present study suggests that a daily treatment of PM containing phytoestrogens exerts the estrogenic effect of suppression on gonadotropin levels in aged menopausal monkeys in a dose dependent manner. After the cessation of PM treatment, the decreased gonadotropin levels can be recovered to the pre-treatment levels within 60 days and depended on doses. Muangman and Chershewasart, (2001) reported that the intake of 200 mg/day of the crude extract of PM improved hot flush, skin dryness, and others without changes in blood cells, liver, and renal function and those symptoms related to high gonadotropin levels (Overlie et al., 2002; Schmidt et al., 2002). From the present study, it can postulate that PM can relief postmenopausal symptoms by suppressive effect on the secretion of gonadotropins.

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